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13. ABSTRACT *(Maximum 200 Words)*

Breast cancer is a genetic disease involving both gain and loss of function mutations in many different genes. It is important to define which genes are significant mutational targets in sporadic breast tumors so that treatments can be directed based on the knowledge of the genetic changes in the tumor. This proposal is focused on identifying tumor suppressor genes which are mutated in sporadic breast cancer using a novel genetic screen. This genetic screen involves applying chromosome engineering technology to delete specific chromosomal regions in mouse mammary epithelial cells *in vivo*, which should allow the induction of tumor suppressor mutations in these haploid regions. We have established that tissue specific expression of cre can recombine *loxP* sites with 10% efficiency even when they are several Megabases apart. We used an existing transgenic cre strain which is expressed in the heart. In order to achieve recombination in mammary epithelial cells we have been developing other cre lines. These include an *MMTV-Cre* line targeted to the *Hprt* locus as well as a line in which Cre has been knocked into the beta-casein locus. We have been evaluating the specificity of these lines using a reporter strain in which *lacZ* is activated following the expression of Cre. When a line with the desired tissue specificity is obtained, tissue specific induced chromosome deletions will be performed with relevant chromosomal regions.

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FOREWORD

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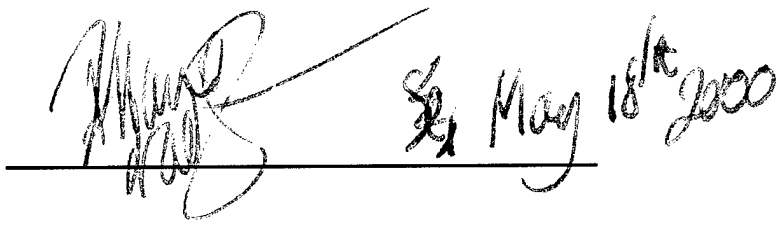

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- **INTRODUCTION**

This proposal is focused on identifying tumor suppressor genes which are mutated in sporadic breast cancer. We are proposing to identify these tumor suppressor genes by a genetic screen *in vivo*. This genetic screen involves the adaptation of our chromosome engineering technology to delete specific chromosomal regions in mouse mammary epithelial cells *in vivo*. We are using developing line of mice which exhibit tissue specific expression of Cre in mammary epithelial cells. We are using these lines to induce long-range deletion events *in vivo*. Tissue specific induced chromosome deletion *in vivo*, when combined with insertional mutagenesis has the power to directly identify tumor suppressor genes mutated in sporadic breast cancer.

(6) BODY

A. Demonstration of long-range recombination (see attached report by Zheng et al., 2000).

B. Demonstration of very long range recombination generating inversion chromosomes for genetic screens (see attached report by Zheng et al., 1999). This is an extension of the original goal, which was pursued because recombination over large distances was much more efficient that expected.

C. Transmission of 3 mammary specific cre-alleles and evaluation of these alleles. This includes a knock-in of *cre* into the beta-casein locus, and two *MMTV-cre* alleles in the *Hprt* locus. These alleles were evaluated following a cross with a reporter line of mice designed to activate Lac Z following a cre-excision event. Thus tissues can simply be stained to visualize and quantify cre activity. Lac Z activity was examined in virgin glands and in during pregnancy. A brief description of these lines follows.

   (i) *Hprt–MMTV-cre allele 1.* This allele is a multiple copy insertion of an MMTV-cre transgene into the *Hprt* locus (achieved by insertional targeting). Staining of mammary glands from this line revealed less than 1% staining, mainly limited to the ducts and a few end buds (Figure 1). Our assessment of this line of mice is that the recombination frequency is currently too low to be practical.

   (ii) *Hprt–MMTV-cre allele 2.* This allele is a single copy insertion of the same vector described above. We are hoping that this single copy allele will be much more useful than allele 1. It is currently being crossed to the reporter and we hope to have Lac Z staining data soon.

   (iii) *ß–casein knock-in.* This allele is illustrated in Figure 2. This consists of a splice-acceptor cre transgene inserted into the first intron of the *ß–casein* locus. This allele was generated by replacement vector targeting and is able to

express cre because of a splicing event from the first exon of casein (which is a non-coding exon). In order to select for the targeted clones we have included a selectable marker (*PGK-Hprt*) which is cloned down stream of the cre-coding sequences.

Our initial assessment of this transgene is that it worked very well, since mammary glands were entirely blue. However further investigations revealed that there were significant complications with this particular allele, because the recombination events appeared to occur during early embryonic development. This "ectopic" activation results in recombination in multiple tissues, which could cause problems because of developmental effects caused by hemizygosity in different tissues.

There are two possible causes of this "ectopic" expression, either there is an unappreciated early expression pattern of casein which has been revealed by the sensitivity of the reporter to cre-expression or cre has been activated in this allele because of the proximity of the *PGK-Hprt* locus. We are currently removing the selection cassette through a second round of gene targeting, this derivative allele will be assessed in the same way as the original allele prior to attempting long range deletions in vivo.

(7) KEY RESEARCH ACCOMPLISHMENTS

- Long range tissue specific *cre* recombination achieved at 10% efficiency *in vivo.*
- Large inversions generated in ES cells at low efficiency and established in mice
- Three germ line mammary cre alleles generated
- Reporter gene evaluation of cre alleles revealed lack of specificity.

(8). REPORTABLE OUTCOMES

Manuscripts:

- Zheng B, Sage M, Sheppeard EA, Jurecic V, and Bradley A (2000). Engineering mouse chromosomes with *Cre-loxP*: Range, efficiency and somatic applications. **Mol. Cell. Biol**, 20, 648-655.
- Zheng B, Sage M, Cai W-W, Thompson DM, Tavsanli BC, Cheah, Y-C, and Bradley A (1999). Engineering a mouse balancer chromosome. **Nature Genetics**, 22, 375-378.

Presentations:

(i) Zheng et al., Thesis defense, September 1999, Title: Manipulating the Mouse Genome: Single Gene Knockouts and Large Chromosomal Rearrangements

(ii) MD Anderson Millennium Symposium, January 9-12, 2000, Title: Tumor Suppressor Genetics: DNA Chips, Genomic Strategies & New Mouse models

(iii)University of Minnesota, March 13, 2000, Title: Uncovering Recessive Oncogenes by Inducing Segmental Haploidy and Elevating Mitotic Recombination Rates in Mice

(iv) Biogen Symposium, December 1, 1999, Title: Contemporary Approaches to Extracting Function from the Mouse Genome

(v) Japan meeting, November 16-18, 1999, Title: Segmental Haploidy Mice and Tumor Suppressor Genetics

(vi) College Station, October 21, 1999, Title: Tumor Suppressor Knockouts: A Gene and Genome Based Analysis

(vii) University of Pennsylvania, May 10, 1999, Title: Tumor Suppressors: How do they do it and how do you find them?

Patents

-none

Degrees.

Dr. Binhai Zheng's Ph.D. work was partially supported by this grant. Dr. Zheng graduated in September 1999.

- **CONCLUSIONS**

It has been established that long range cre-loxP recombination is efficient in vivo. However, the desired specificity of the Cre has not yet been obtained. It is probably not a serious problem that the cre lines also recombine during embryogenesis provided that the large deletions induced do not cause problems of haplo-insufficiency. During the next budget period we shall attempt to improve the specificity of the cre line by removing a selectable maker present in the locus. We will also attempt some mammary specific recombination with the existing lines to determine whether the lack of specificity will cause any developmental problems.

If mammary specific long-range recombination is achieved at 10% frequencies as we expect, them we will have established a very powerful genetic system for isolating mammary specific tumor suppressor genes.

FIGURE 1

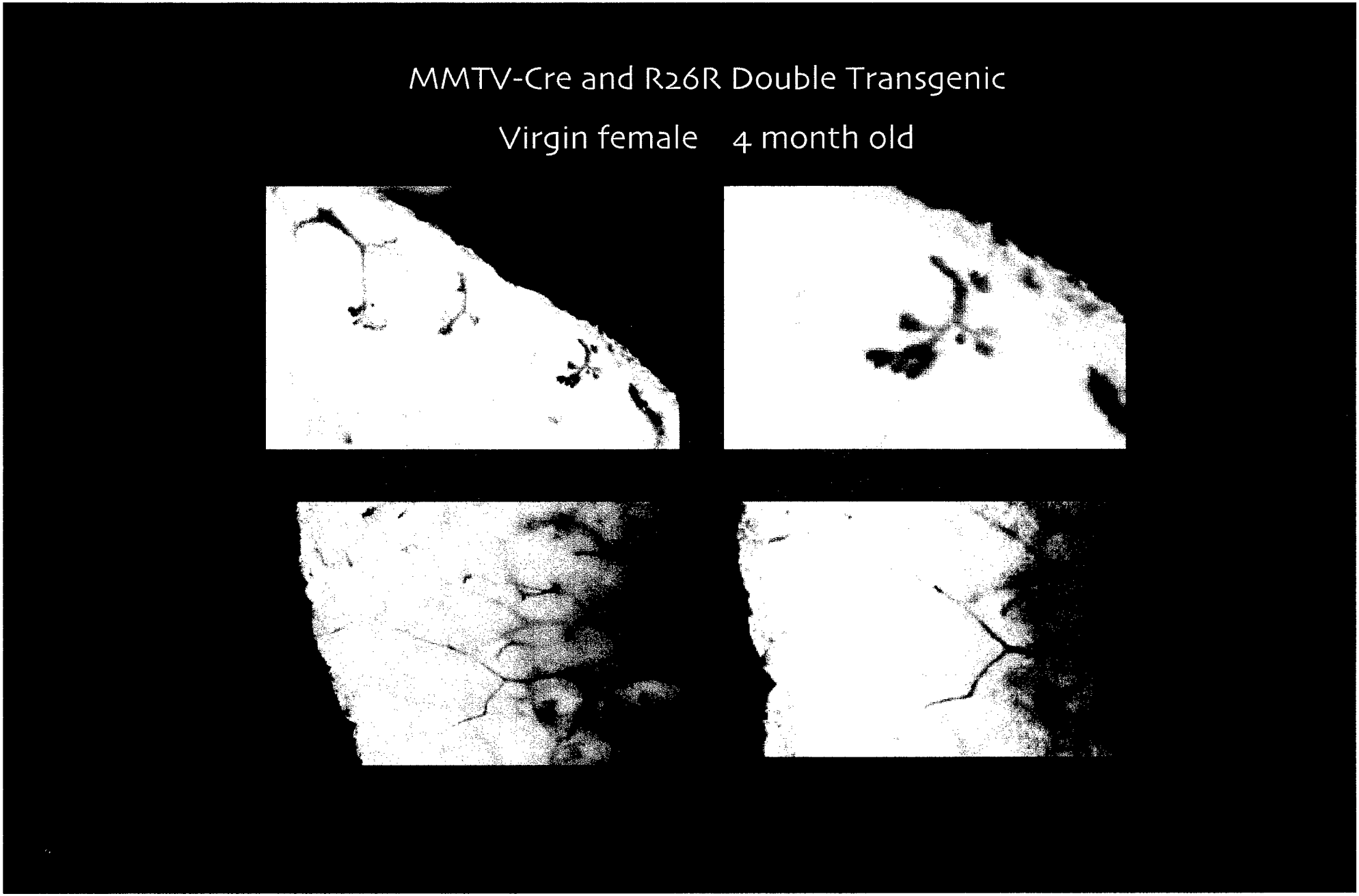
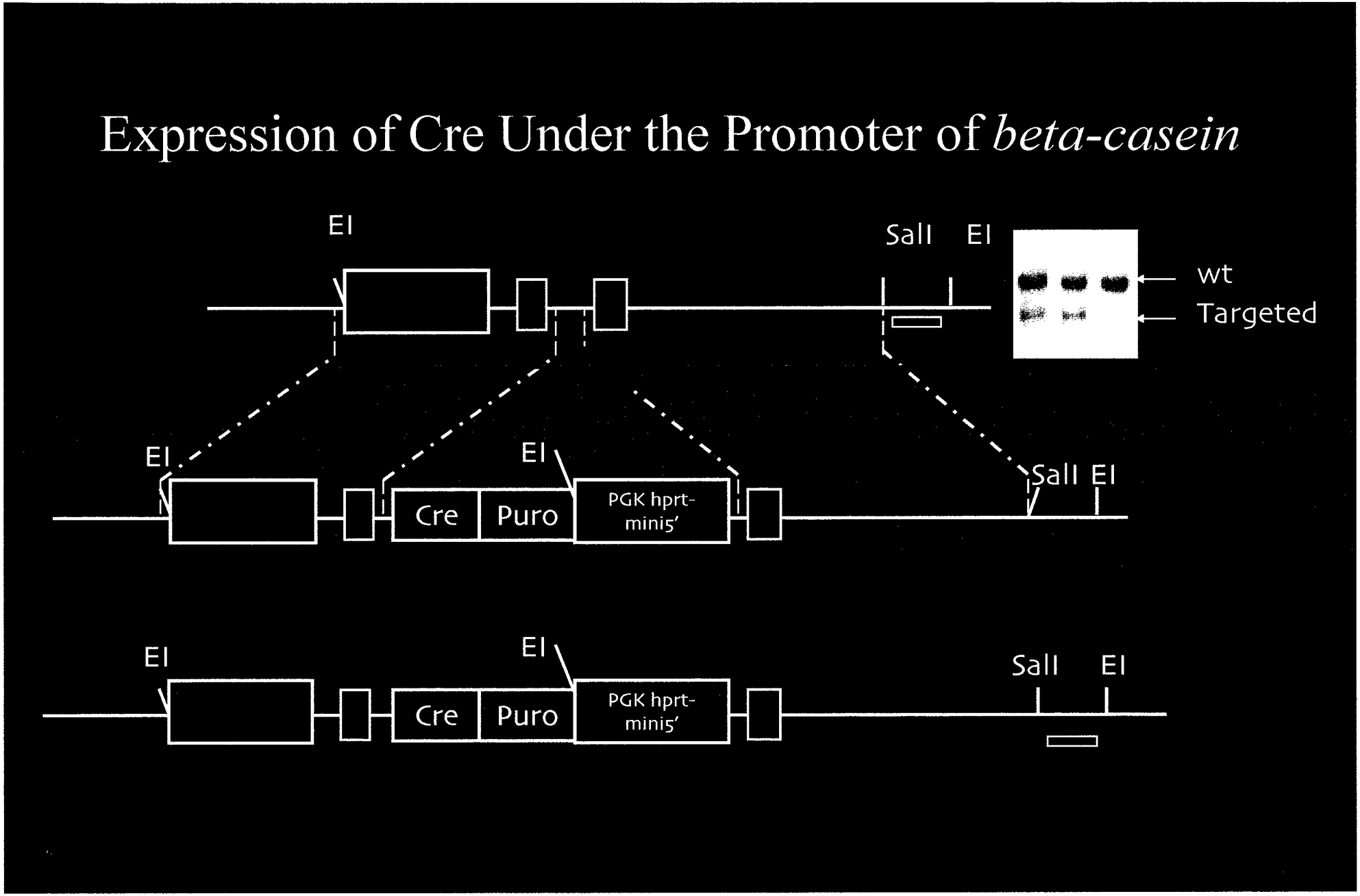

MMTV-Cre and R26R Double Transgenic
Virgin female 4 month old

FIGURE 2


Expression of Cre Under the Promoter of *beta-casein*
EI
SalI EI
wt
Targeted
EI
EI
SalI EI
Cre
Puro
PGK hprt-mini5'
EI
EI
SalI EI
Cre
Puro
PGK hprt-mini5'

Engineering a mouse balancer chromosome

Binhai Zheng[1], Marijke Sage[1], Wei-Wen Cai[1], Debrah M. Thompson[1,2], Beril C. Tavsanli[3], Yin-Chai Cheah[1] & Allan Bradley[1,2,3]

Balancer chromosomes are genetic reagents that are used in *Drosophila melanogaster* for stock maintenance and mutagenesis screens[1]. Despite their utility, balancer chromosomes are rarely used in mice because they are difficult to generate using conventional methods. Here we describe the engineering of a mouse balancer chromosome with the Cre-*loxP* recombination system. The chromosome features a 24-centiMorgan (cM) inversion between *Trp53* (also known as *p53*) and *Wnt3* on mouse chromosome 11 that is recessive lethal and dominantly marked with a *K14-Agouti* transgene[2]. When allelic to a wild-type chromosome, the inversion suppresses crossing over in the inversion interval, accompanied by elevated recombination in the flanking regions. The inversion functions as a balancer chromosome because it can be used to maintain a lethal mutation in the inversion interval as a self-sustaining *trans*-heterozygous stock. This strategy can be used to generate similar genetic reagents throughout the mouse genome. Engineering of visibly marked inversions and deficiencies is an important step toward functional analyses of the mouse genome and will facilitate large-scale mutagenesis programs.

Balancer chromosomes enable the maintenance of lethal mutations without selection in *Drosophila*[3]. The rationale is as follows: for a lethal mutation in gene *A*, *lA*, an *lA*/+ × *lA*/+ cross will yield progeny in the ratio of 1 *lA*/*lA* (inviable):2 *lA*/+:1 +/+. Because *lA*/+ and +/+ progeny are often indistinguishable, maintaining *lA* requires constant selection or genotyping. By introducing another lethal mutation, *lB*, to the homologous chromosome, *lA* is maintained in a *trans*-heterozygous state, *lA* +/+ *lB*. An *lA* +/+ *lB* × *lA* +/+ *lB* cross will always produce *lA* +/+ *lB* progeny so long as there is no recombination between the two loci (*lA* +/*lA* + and + *lB*/+ *lB* progeny will also be produced, but are inviable). To prevent recombination from occurring, a crossover suppressor must be present. This is most conveniently achieved by an inversion (or a complex of inversions) on the chromosome carrying *lB* (the balancer), because a single crossover between an inversion and a normal chromosome gives inviable dicentric and acentric products or aneuploidy[4] (Fig. 1). A self-perpetuating *lA* +/+ *lB* stock constitutes a balanced lethal system. In addition to *lB*, the inversion is typically marked with a dominant marker so that progeny carrying the balancer are readily identified. Because balancer chromosomes suppress recombination, they are used to maintain the integrity of mutagenized chromosomes and are therefore crucial reagents for mutagenesis screens[1]. For instance, in intercrosses between siblings that have inherited the same balancer chromosome and the mutagenized chromosome, absence of non-balancer–carrying progeny (as assessed by the dominant marker) indicates the presence of one or more recessive lethal mutations on the mutagenized chromosome.

In the mouse, advances in *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis[5–7] and positional cloning methods[8,9] have presented large-scale mutagenesis as a viable approach for functional genomics. Such phenotype-driven mutagenesis screens make no assumption about the gene products to be analysed and therefore will identify novel pathways, novel genes and novel functions of known genes. A model study at a region surrounding the mouse albino locus has yielded a wealth of functional genomic information for this region[10–12]. Most regions of the mouse genome are not accessible for such analyses, however, due to a lack of marked chromosomal rearrangements. In this study, we tested the idea of engineering a marked inversion as a balancer chromosome in the mouse.

We made a 24-cM inversion between *Trp53* and *Wnt3* on mouse chromosome 11. The conserved linkage to a gene-rich segment on human chromosome 17 (ref. 13) makes this region a suitable target for large-scale mutagenesis efforts. We chose *Wnt3* as an endpoint because a *Wnt3* mutation confers homozygous lethality[14]. We chose the genetic distance to be sufficiently large to be useful for a considerable region of chromosome 11 (24 cM/80 cM≅30%), and sufficiently small to minimize double crossovers that obviate the balancing effect. We used the Cre-*loxP* chromosome engineering strategy[15] to generate the inversion in mouse embryonic stem (ES) cells by successive gene targeting of a *loxP* site to the two endpoints, followed by Cre-mediated recombination between the two *loxP* sites leading to the desired

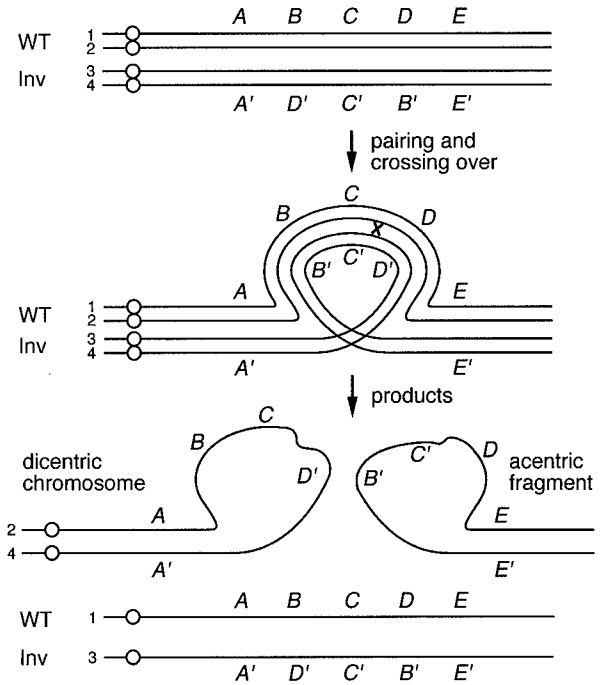


Fig. 1 How a single crossover between a wild-type chromosome and a paracentric inversion (an inversion that does not span the centromere) leads to inviable dicentric and acentric products. WT, wild type; Inv, inversion. The four chromatids are numbered. '×' indicates the site of a crossover. Adapted from ref. 4.

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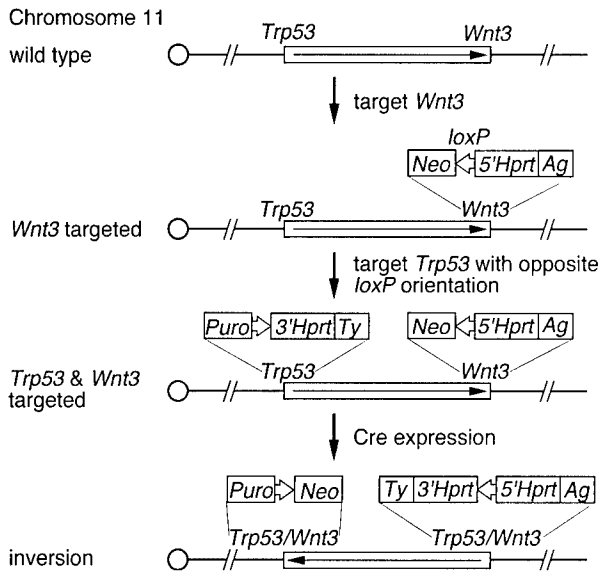

Fig. 2 A three-step Cre-*loxP*-based strategy to engineer the *Trp53-Wnt3* inversion. *Neo*, neomycin resistance gene; *Puro*, puromycin resistance gene; *Ag*, *K14-Agouti* transgene; *Ty*, tyrosinase minigene; 5′ *Hprt* or 3′ *Hprt*, 5′ or 3′ half of an *Hprt* minigene. For simplicity the other chromosome 11 homologue (wild type) is not shown.

rearrangement (Fig. 2). Reconstitution of a full-length *Hprt* gene from the two non-functional halves that are linked to either *loxP* site provides a selection for the recombination products in HAT medium[15]. By incorporating coat-colour markers into the targeting vectors, the inversion is visibly marked. We targeted *Wnt3* with a dominant *K14-Agouti* coat-colour gene that has been shown to confer a yellowing of the coat colour on an otherwise wild-type agouti mouse[2,16]. We targeted *Trp53* with a tyrosinase (*Tyr*) minigene that has been shown to give pigment to an otherwise albino mouse[17].

We targeted both *Wnt3* and *Trp53* with replacement vectors designed to inactivate the genes (Fig. 3). The targeted *Wnt3* allele, $Wnt3^{Brdm2}$, was constructed to orient the 5′ *Hprt-loxP* cassette toward the centromere[18] (Fig. 3*d*). As the transcript orientation of *Trp53* relative to the centromere was unknown, we constructed two targeting vectors for *Trp53* with opposite *loxP*-site orientations (Fig. 3*a*, and data not shown). The targeted *Trp53* alleles, $Trp53^{Brdm2}$ and $Trp53^{Brdm3}$, were obtained in heterozygous $Wnt3^{Brdm2}$ ES cell lines. We electroporated six $Trp53^{Brdm2}$-$Wnt3^{Brdm2}$ double-targeted cell lines with a Cre expression plasmid and obtained HAT-resistant colonies from five of these cell lines ($6.0\pm0.4\times10^3$ (mean±s.e.m.) HAT-resistant colonies per 10^7 electroporated cells). This suggested that the HAT-resistant colonies were inversions resulting from recombination between two *loxP* sites on the same chromosome in opposite orientations, whereas the remaining parental cell line carried the two *loxP* sites on different chromosome homologues leading to inviable dicentric and acentric chromosomal fragments upon Cre recombination[15]. Drug-resistance tests indicated that the HAT-resistant colonies were resistant to both neomycin and puromycin, as expected for inversion products. Southern-blot analysis using probes at either junction region detected the expected restriction fragments (Fig. 3), indicating that a precise rearrangement had occurred. Fluorescence *in situ* hybridizations (FISH) on metaphase chromosomal spreads confirmed that an inversion

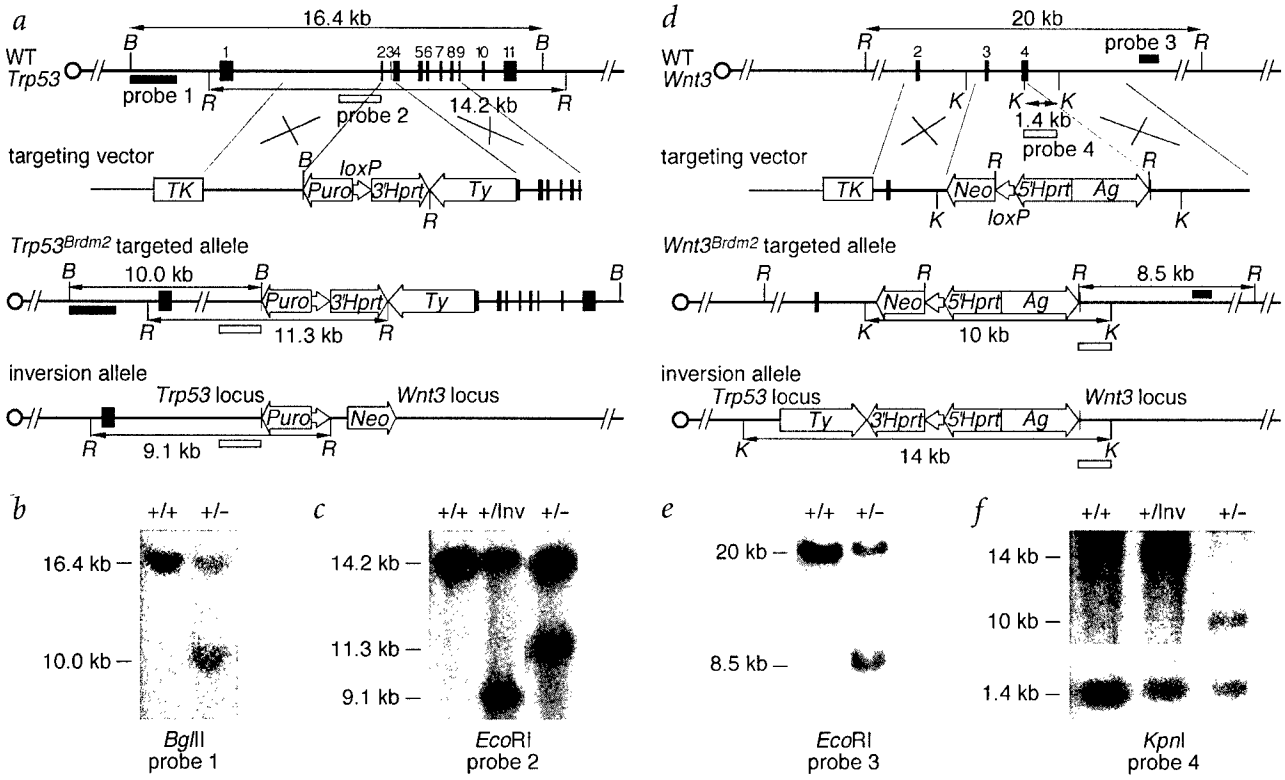

Fig. 3 Southern-blot analysis of the $Trp53^{Brdm2}$ targeted allele, the $Wnt3^{Brdm2}$ targeted allele and the *Trp53-Wnt3* inversion. ***a***, Genomic structure of wild-type *Trp53* locus, targeting vector, predicted $Trp53^{Brdm2}$ targeted allele and inversion allele. Exons are indicated by vertical black bars. *TK*, herpes simplex virus thymidine kinase gene; *Neo*, neomycin resistance gene; *Ty*, tyrosinase minigene; *Puro*, puromycin resistance gene; *Ag*, *K14-Agouti* transgene. *B*, *Bgl*II; R, *Eco*RI, K, *Kpn*I. ***b***, Southern-blot analysis to detect the $Trp53^{Brdm2}$ allele using probe 1. ***c***, Southern analysis to distinguish between wild-type, targeted and inversion alleles using probe 2. ***d***, Genomic structure of wild-type *Wnt3* locus, targeting vector, predicted $Wnt3^{Brdm2}$ targeted allele and inversion allele. ***e***, Southern-blot analysis to detect the $Wnt3^{Brdm2}$ allele using probe 3. ***f***, Southern analysis to distinguish between wild-type, targeted and inversion alleles using probe 4.

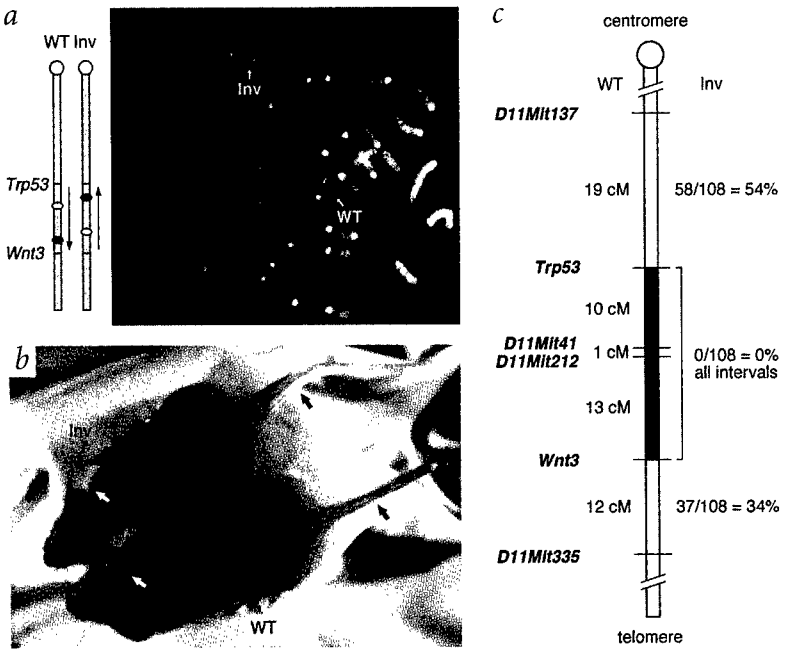

Fig. 4 Characterization of the *Trp53-Wnt3* inversion. ***a***, FISH analysis of the *Trp53-Wnt3* inversion. WT, wild type; Inv, inversion. Yellow, a *D11Mit320* BAC probe; red, a *D11Mit263* BAC probe. The parental double-targeted cell lines showed a wild-type order of the two probes on both chromosome 11 homologues (data not shown). ***b***, Expression of the dominant marker *K14-Agouti* in the *Trp53-Wnt3* inversion. The back of the earlobes and the tails are indicated with open and filled arrows, respectively. ***c***, Recombination frequencies in and around the *Trp53-Wnt3* inversion. The established genetic distances in wild type are marked in cM (left). The observed recombination frequencies with the inversion are shown (right). The inversion is indicated by a filled bar.

was obtained (Fig. 4*a*). In contrast, the $Trp53^{Brdm3}$-$Wnt3^{Brdm2}$ double-targeted cell lines gave rise to duplication/deletion products, as expected for recombination between two *loxP* sites in the same orientation (data not shown).

We next transmitted ES cells harbouring the *Trp53-Wnt3* inversion, In(11*Trp53*; 11*Wnt3*)8Brd, through the mouse germ line via standard procedures[19]. Chimaeric males were mated with C57BL/6-$Tyr^{c\text{-}Brd}$ (albino) females. Among the (agouti) germline pups, approximately one-half were wild-type agouti and the other half had lighter tail and ear colouring (Fig. 4*b*), which we confirmed to be due to the expression of the *K14-Agouti* transgene. Except for the coat-colour difference, inversion progeny were phenotypically indistinguishable from wild-type littermates. Preliminary studies on homozygous inversion embryos indicate they have the same phenotype as *Wnt3*-null mutants[14].

To test the expression and transmission of the coat-colour markers, we backcrossed (129S7×C57BL/6-$Tyr^{c\text{-}Brd}$) F_1 heterozygous inversion males (heterozygous for the albino and agouti loci) to three different inbred strains: 129S5 (agouti), C57BL/6J (black) and C57BL/6-$Tyr^{c\text{-}Brd}$ (albino). In all cases, a lighter tail colour co-segregated with the inversion (as determined by Southern-blot analysis) in an agouti or black background (Table 1). Thus, *K14-Agouti* functions as a dominant marker in a non-albino background. Among the albino progeny from the C57BL/6-$Tyr^{c\text{-}Brd}$×F_1 matings, there was no detectable difference in coat colour between the inversion mice and wild-type littermates, indicating that the *Tyr* minigene on the inversion chromosome was not expressed.

To examine whether the inversion suppresses crossing over in the inversion interval, we determined the recombination frequencies on chromosome 11 by scoring the backcross progeny. In addition to Southern-blot analysis with *Trp53* and *Wnt3* probes at the inversion junctions (Fig. 3), we analysed four polymorphic SSLP markers that lie proximal (*D11Mit137*), internal (*D11Mit41*, *D11Mit212*) and distal (*D11Mit335*) to the inversion (Fig. 4*c*). The two internal markers map close to the centre of the inversion and were used to screen for double crossovers. Among all 108 meioses examined, there was no recombination within the inversion, indicating successful suppression of recombination (Fig. 4*c*). Because of chiasma interference, the frequency of double crossovers is presumably lower than 1%. In contrast, the flanking region showed elevated recombination frequencies (Fig. 4*c*), possibly as a mechanism to compensate for the loss of recombination in the inversion interval[20].

To test whether the inversion indeed functions as a balancer chromosome, we crossed the inversion to a recessive lethal mutation, $Hoxb4^{r}$ (ref. 21), which lies in the inversion interval. We intercrossed mice *trans*-heterozygous for the inversion and the $Hoxb4^{r}$ mutation, collected the progeny and genotyped them at weaning age. Of four litters from two matings, all were heterozygous for both the inversion and the $Hoxb4^{r}$ mutation (Fig. 5). The average litter size was four, as expected if one-half the pups were inviable.

Our results demonstrate that large inversions compatible with normal development can be engineered in ES cells and established in the mouse. The *Trp53-Wnt3* inversion can be used to maintain any recessive lethal, sterile or other detrimental mutations in the 24-cM interval, such as mutations in *Brca1* or the *Hoxb* series. It can be used in chromosome 11 region-specific mutagenesis screens[22]. To create a balancer for the entire chromosome, multiple inversions must be generated with a combination of strategies such as mutant *loxP* sites[23,24] or a different site-specific recombination system[25,26]. The strategy described here can also be used to engineer marked deficien-

Table 1 • Coat-colour segregation of inversion backcrosses

Parental genotypes (female×male)	Agouti progeny		Black progeny		Albino progeny	
	WT normal tail	Inv light tail	WT normal tail	Inv light tail	WT	Inv
129S5×F_1(Inv)	18	21	–	–	–	–
C57BL/6J×F_1(Inv)	12	4	9	5	–	–
C57BL/6-$Tyr^{c\text{-}Brd}$×F_1(Inv)	9	3	4	3	11	9

The genotypes at the albino locus (or *Tyr*; *C*) and the agouti locus (*A*) are: *CCAA* for 129S5; *CCaa* for C57BL/6J; *ccaa* for C57BL/6-$Tyr^{c\text{-}Brd}$. 129S7 and 129S5 are identical, except at the *Hprt* locus. F_1 refers to (129S7×C57BL/6-$Tyr^{c\text{-}Brd}$) F_1.

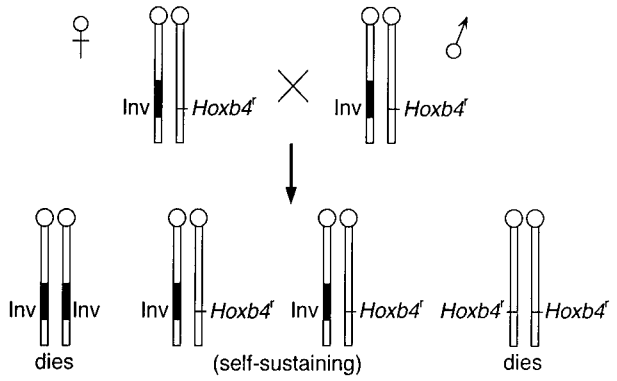

Fig. 5 Use of the *Trp53-Wnt3* inversion to balance the *Hoxb4*^{r} lethal mutation.

cies. The ability to engineer marked deficiencies and balancer chromosomes will greatly facilitate genetic studies in the mouse.

Methods

Generation of the *Trp53-Wnt3* inversion mice. We constructed the *Wnt3* targeting vector by modifying a previous vector[18] such that correct targeting would replace a 2.1-kb fragment containing exon 3 and most of exon 4 with a cassette containing a neomycin resistance gene, a *loxP* site, 5′ *Hprt* (also known as *hprtΔ3′*; ref. 15) and the *K14-Agouti* transgene[2]. We generated chimaeric mice from two *Wnt3*^{Brdm2} targeted ES cell lines, both of which gave germline transmission. The two cell lines behaved the same in later experiments and are therefore not distinguished in the text. To obtain isogenic genomic DNA to construct *Trp53* targeting vectors, we isolated a clone from an arrayed 129S5 genomic phage library using primer pairs amplifying a fragment of *Trp53* that contains an intron to distinguish between *Trp53* and its pseudogene[27]. A phage clone was obtained, mapped and found to contain exons 1–9. Another *Nco*I site lies 4 kb upstream of the *Nco*I site in exon 2 that is absent in a published map[28], possibly due to a polymorphism. We constructed a replacement vector with a selection cassette containing a puromycin-resistance gene, a *loxP* site, 3′ *Hprt* (after correcting a mutation in the coding sequence of the original cassette, B.Z. and A.B., manuscript submitted; also known as *hprtΔ5′*; ref. 15) and the *Tyr* minigene[17] using the 4-kb *Nco*I fragment as the 5′ homology arm and a 2.6-kb *Sac*I fragment as the 3′ homology arm (Fig. 3*a*). Correct targeting was expected to delete a 0.6-kb genomic fragment between the *Nco*I site in exon 2 and the *Sac*I site in exon 4. We constructed a second replacement vector with the entire selection cassette in the reverse orientation (data not shown).

We performed ES cell cultures, electroporation, mini-Southern-blot analysis on ES cell colonies and generation of chimaeric and germline mice as described[19,29]. We used AB2.2 ES cells that were derived from a 129S7 male embryo. For Cre expression, we electroporated 10^7 ES cells with plasmid pOG231 (25 μg) and initiated HAT selection after 48 h (ref. 15). Drug-resistance tests on Cre recombination products were performed as described[15,18]. We introduced the *Hoxb4*^{r} mutation from a 129S5 inbred background.

Fluorescence *in situ* hybridization. We prepared metaphase chromosome spreads from ES cells as described[30]. FISH was performed following a standard protocol[31]. Two BAC clones that map within the inversion were differentially labelled and used as the probe.

PCR polymorphic analysis. We used four SSLP markers[8] polymorphic between 129S5 and C57BL/6 (H. Su and A.B., unpublished data). The products were analysed on a 4% agarose gel (3:1 high resolution blend, Amresco). The sizes of the amplified products are (in the order of 129S5 or 129S7, C57BL/6): *D11Mit137*, 152 bp, 138 bp; *D11Mit41*, 158 bp, 136 bp; *D11Mit212*, 164 bp, 148 bp; *D11Mit335*, 134 bp, 120 bp. The genetic distances on the wild type chromosome were derived from chromosomal positions (in cMs) in the Chromosome Committee Reports (http://www.informatics.jax.org/bin/ccr/index).

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Engineering Mouse Chromosomes with Cre-*loxP*: Range, Efficiency, and Somatic Applications

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Chromosomal rearrangements are important resources for genetic studies. Recently, a Cre-*loxP*-based method to introduce defined chromosomal rearrangements (deletions, duplications, and inversions) into the mouse genome (chromosome engineering) has been established. To explore the limits of this technology systematically, we have evaluated this strategy on mouse chromosome 11. Although the efficiency of Cre-*loxP*-mediated recombination decreases with increasing genetic distance when the two endpoints are on the same chromosome, the efficiency is not limiting even when the genetic distance is maximized. Rearrangements encompassing up to three quarters of chromosome 11 have been constructed in mouse embryonic stem (ES) cells. While larger deletions may lead to ES cell lethality, smaller deletions can be produced very efficiently both in ES cells and in vivo in a tissue- or cell-type-specific manner. We conclude that any chromosomal rearrangement can be made in ES cells with the Cre-*loxP* strategy provided that it does not affect cell viability. In vivo chromosome engineering can be potentially used to achieve somatic losses of heterozygosity in creating mouse models of human cancers.

Specific chromosomal rearrangements can be engineered in mice to model human chromosomal disorders, such as those associated with deletions or duplications of chromosomal segments (for example, Smith-Magenis syndrome, Downs syndrome, and Charcot-Marie-Tooth type 1A) (5, 7, 10). Chromosomal rearrangements also facilitate genetic studies (2, 14). Inversion chromosomes can be used to establish balanced lethal systems to facilitate stock maintenance. Deletions can be used for mapping and in genetic screens for recessive mutations.

In *Drosophila melanogaster* there is a wealth of chromosomal rearrangements that are widely used as genetic tools. In particular, chromosomal deletions (deficiencies) which collectively cover approximately 60 to 70% of the genome have been indispensable in mapping recessive mutations and in region-specific mutagenesis screens. The use of deletions in mice, however, has been much more limited because of the paucity of chromosomal deletions which, until recently, were restricted to a few regions of the mouse genome flanking visible genetic markers (14). The application of the Cre-*loxP* recombination system over large distances in mouse embryonic stem (ES) cells has made it possible to engineer specific chromosomal rearrangements in the mouse (13, 17). This chromosome engineering strategy involves three manipulation steps in ES cells (see Fig. 1): (i) one *loxP* site is targeted to one endpoint along with the 5′ half of an *Hprt* selectable marker gene (5′ *hprt*); (ii) another *loxP* site is targeted to a second endpoint with the 3′ half of the *Hprt* gene (3′ *hprt*); and (iii) transient expression of Cre recombinase catalyzes *loxP* site-specific recombination, leading to the desired rearrangement. Reconstitution of a full-length *Hprt* gene provides selection for ES cells with the recombination products in culture in HAT (hypoxanthine-aminopterin-thymidine) medium. By using this technology, deletions, duplications, inversions, or translocations can be generated depending upon the relative position and orientation of the two *loxP* sites and selection cassettes (13, 17).

The Cre-*loxP* chromosome engineering strategy provides a unique and unprecedented opportunity to manipulate the mouse genome. However, several critical questions remain to be answered in order to explore fully the potential of this technology. First, is there any limit as to the kind and size of rearrangements that can be made with this technology? While there are likely to be biological limits in mice, ES cells harboring large chromosomal deletions offer an opportunity to perform haploid genetic screens in vitro. For such applications, the larger the deletion, the more powerful the screen. Second, what is the efficiency of Cre-mediated recombination for substrates of different genetic distances? This will be pertinent to the scope and applicability of this technology. Third, can this strategy be used to engineer chromosomes somatically, that is, in a tissue- or cell-type-specific manner without the strong positive selection schemes that are used in cell culture? Tissue-specific deletions also enable recessive genetics to be employed somatically, for instance, to induce loss of heterozygosity (LOH) to model genetic changes in human cancers or to conduct screens for novel tumor suppressor genes in combination with mutagenesis strategies. Somatically induced deletions may avoid the developmental problems associated with larger germline deletions and consequently a larger chromosomal region can be studied in a single animal.

To address these questions, we applied the Cre-*loxP* chromosome engineering strategy to various parts of mouse chromosome 11 (Chr 11) in ES cells and in vivo. With an improved selection cassette, we obtained an 11% deletion efficiency for a two-centimorgan (2-cM; equivalent to 4 Mb) deletion substrate in murine ES cells. Rearrangements of up to three-quarters of Chr 11 have been made, demonstrating that there appears to be no recombination-based restriction as to what type of rearrangements can be made provided that ES cells tolerate the genetic change. We found that the efficiency of Cre-mediated recombination between two *loxP* sites on the

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same chromosome (*cis*) decreases with increasing genetic distance. We found that large chromosomal deletions may be deleterious to ES cells and that deletions which were lethal to developing embryos could be engineered somatically at high efficiencies, breaking ground for somatic chromosome engineering.

MATERIALS AND METHODS

Construction of targeting vectors. The *Hsd17b1* targeted cell line has been described elsewhere (13). The targeting vectors for *Wnt3* (modified from a previous version [8]) and *p53* have also been described elsewhere (24). All microsatellite markers were targeted with insertion vectors. The targeting vectors for *D11Mit199* and *D11Mit69* were modified from previous versions (8), replacing the mutant 3′ *hprt* cassette with the wild-type sequence.

The *D11Mit142* and *D11Mit71* loci were targeted with insertion vectors generated from a targeting-ready genomic library that contains the puromycin resistance gene, a *loxP* site, 3′ *hprt* cassette, and an agouti coat color transgene in the vector backbone (23). Clones isolated from this library were restriction mapped, and a gap was created in the region of homology which was used as the probe to detect targeting by Southern analysis. The targeting vectors for *D11Mit142* have been described (23). A clone with a 10.9-kb genomic insert at *D11Mit71* was isolated from the 3′ *hprt* library and mapped with several restriction enzymes. The insert consists of two flanking (2.3 and 3.9 kb) and three internal (0.8, 3.3, and 0.6 kb) *Nco*I fragments. The internal fragments were deleted from the clone to create a gap in the region of homology, resulting in targeting vector pTV*D11Mit71*F. The insert was then flipped by using rare cutter *Asc*I sites that flank the insert, resulting in targeting vector pTVD11Mit71R which was used to deliver the *loxP* site to the *D11Mit71* locus with the reverse orientation. The 3.3-kb internal *Nco*I fragment was used as the probe in mini-Southern analysis to detect gap repair-dependent targeting events (20, 23). This probe hybridizes to a 6-kb and a (weak) 2.8-kb wild-type *Eco*RI fragment and, in targeted clones, an additional 18.6-kb targeted fragment resulting from the insertion of the vector sequence into the targeted locus.

Generation and analysis of chromosomal rearrangements. ES cell cultures, gene targeting, and germ line transmission were performed as described previously (12). AB2.2 ES cells were used in most experiments except in a few cases where a hybrid ES cell line (between 129S7 and C57BL/6-*Tyr*$^{c\text{-}Brd}$), ER3.4, was used (E. Regel and A. Bradley, unpublished data). Electroporation of the Cre expression plasmid pOG231 (11), selection of Cre recombination products with HAT medium, and drug (neomycin and puromycin) resistance tests were performed as described earlier (8, 13) with some modifications. ES cells (80% confluent) were passaged 1 day before electroporation and fed with medium 2 h before electroporation. The cells were then trypsinized and resuspended in phosphate-buffered saline (PBS), and cell counting was performed with a Coulter Counter. The cells were again suspended in PBS to make the final cell density of 1.1×10^7 cells/ml. In a typical transient Cre expression experiment, 25 μg of pOG231 (prepared by CsCl centrifugation, unlinearized) was electroporated into 10^7 ES cells in 0.9 ml of PBS. The electroporation was conducted with a Bio-Rad GenePulser and a Gene Pulser cuvette with a 0.4-cm electrode gap at 230 V and 500 μF. Cells (in PBS) were then mixed with M15 medium and plated on two to three plates at different densities. For the *cis* 2-cM substrates, electroporated cells were subject to serial dilution before plating to enable counting of the HAT-resistant colonies. HAT selection was initiated about 48 h after electroporation, maintained for 8 days and released in hypoxanthine thymidine (HT) for 2 days before the colonies were counted and picked. In all experiments a 10^4 dilution was also plated for each cell line under no selection to count and calculate the number of colonies that survived electroporation. Assessed by this procedure, usually about one-third of the cells undergoing electroporation survived and formed colonies on feeder plates in M15 medium. To control between different experiments, a 2-cM double-targeted cell line was included in each experiment as a control for the Cre recombination efficiency, and this efficiency (~11%) has been consistent throughout all of the experiments.

FISH. Metaphase chromosome spreads from ES cells were prepared as described previously (15). Fluorescence in situ hybridization (FISH) was performed with phage or BAC probes according to a standard protocol (3). The *mPer1* phage clone, BAC 330H2, was labeled with digoxigenin and detected by anti-digoxigenin-rhodamine antibody. BAC 293C22 and BAC 330P14 were labeled with biotin and detected with fluorescein isothiocyanate-avidin. BAC 232M23 was labeled with a mixture of digoxigenin and biotin. The chromosomes were stained with DAPI (4′,6′-diamidino-2-phenylindole). The images were taken as monotonic pictures, and the composites were made with artificial coloration for clarity.

PCR and sequence analysis. Primer P_a, 5′-AGG ATG TGA TAC GTG GAA GA (*Hprt* intron, forward), and primer P_b, 5′-GCC GTT ATT AGT GGA GAG GC (polymerase II promoter in the neomycin resistance gene, reverse), were used to specifically amplify by PCR a fragment containing exon 3 sequence in the 5′ *hprt* cassette. Primer P_a and primer P_c, 5′-CCA GTT TCA CTA ATG ACA CA (*Hprt* exon 9, reverse), were used to specifically amplify exon 3 sequence in the 3′ *hprt* cassette. Primer P_d, 5′-GCA TTG TTT TGC CAG TGT C (*Hprt* exon 6, reverse), was used to sequence exon 3 in the PCR products.

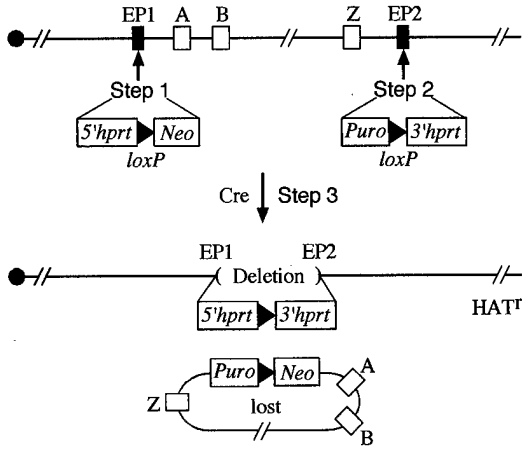

FIG. 1. The Cre-*loxP* based chromosome engineering strategy. 5′ *hprt* was previously named *hprtΔ3′*; 3′ *hprt* was previously name *hprtΔ5′*. A neomycin (*Neo*) or a puromycin (*Puro*) resistance gene is linked to the first or the second *loxP* site, respectively, for positive selection during gene targeting. In this case, Cre recombination between two *loxP* sites targeted in the same orientation in *cis* (on the same chromosome) leads to a deletion that is neomycin and puromycin sensitive due to the loss of the *Neo*- and *Puro*-carrying reciprocal product, a ring chromosome in G_1 (shown) or a duplication sister chromatid in G_2 (not shown). If the two *loxP* sites are on the two different chromosome homologues (in *trans*), a deletion and a duplication will be produced. The rearrangements can then be transmitted through the mouse germline if viable.

The primers used to detect the cardiac specific 2-cM deletion were P1, 5′-CCT CAT GGA CTA ATT ATG GAC (*Hprt* exon 2, forward), and P2, the same as P_c (*Hprt* exon 9, reverse). The primer pair used to detect the αMyHC-Cre (Cre coding sequence under the control of α-myosin heavy chain promoter) transgene has been described elsewhere (1).

RESULTS

High-efficiency Cre-*loxP* based chromosomal engineering with an improved vector in mouse ES cells. Sequence analysis identified a frameshift mutation in the coding portion of the 3′ *hprt* selection cassette (Fig. 2) previously successfully used for chromosome engineering (13), leading to a translation stop codon nine codons downstream of the mutation (Fig. 2A). This mutation should render a reconstituted *Hprt* minigene nonfunctional, yet HAT-resistant colonies were obtained with this cassette. These may have resulted from a repair event during or following Cre recombination (see below). Since the events we have scored to date required selection, the efficiency of Cre-mediated *loxP* site-specific recombination on multimegabase substrates may be greater than that scored by the number of selected HAT-resistant clones. Because the recombination efficiency is pertinent in applications of the Cre-*loxP*-based chromosome engineering strategy, we reassessed this efficiency for a 2-cM interval between *Hsd17b1* (*E$_2$DH*) and *D11Mit199* on Chr 11 (8) by using cassettes without the frameshift mutation. The *D11Mit199* locus was retargeted with the corrected 3′ *hprt* cassette in an ES cell line that had been targeted at the *Hsd17b1* locus with the 5′ *hprt* cassette (13) so that the *loxP* sites were in the same orientation (8). The double-targeted cell lines were electroporated with a Cre expression plasmid (pOG231) (11) or a control plasmid (TyBS) (22), and the recombination efficiency was assessed (defined here as the number of HAT-resistant colonies per cell surviving electroporation). No HAT-resistant colony was obtained with the control plasmid. With the Cre expression plasmid, approximately half of the double-targeted clones yielded recombination efficiencies of approximately 11%, while the rest had ef-

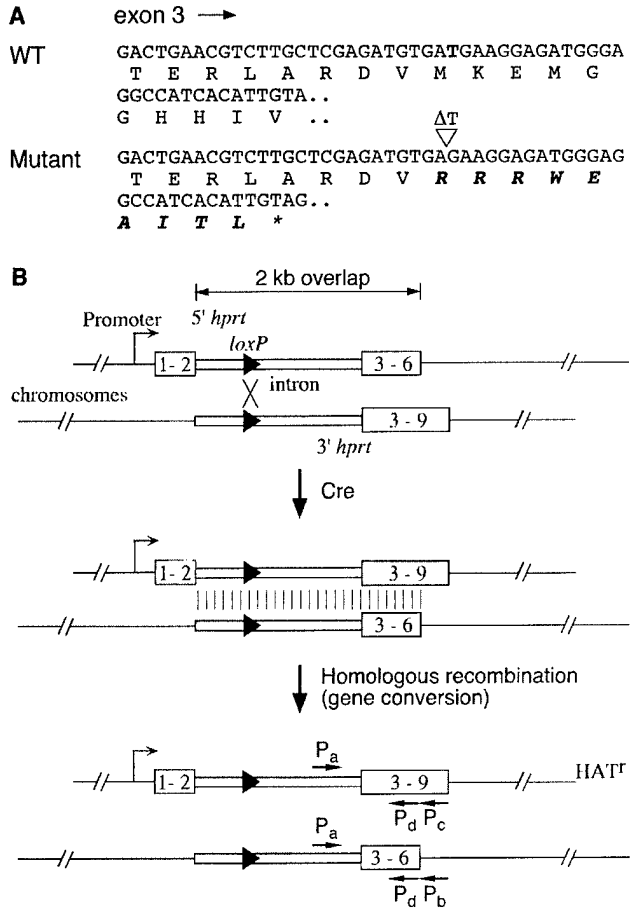

FIG. 2. A frameshift mutation in the original 3′ *hprt* cassette used in chromosome engineering. (A) Partial exon 3 sequence of the wild-type (WT) and mutant 3′ *hprt* cassette with conceptual translation. The thymidine residue in the wild-type sequence that is deleted in the mutant is in boldface and underlined. The altered amino acid residues affected by the mutation is in italicized boldface. *, Stop codon. (B) A proposed mechanism by which HAT-resistant colonies were obtained by a combination of Cre-*loxP* site-specific recombination and homologous recombination, as shown for Cre recombination between two *loxP* sites in *trans* in the same orientation that leads to a deletion and a duplication. 1–2, 3–6, and 3–9 refer to *Hprt* exons. P_a, P_b, P_c, and P_d, primers for PCR and sequence analysis of exon 3 of the *Hprt* gene. The portion of the coding sequence affected by the mutation in exons 3–9 is shaded. The polyadenylation signal and neomycin and puromycin resistance genes are not shown for simplicity.

ficiencies of approximately 0.047% (Table 1). Cre recombination products from the former group are puromycin sensitive (indicating that these cell lines have a deletion), while those from the latter group are puromycin resistant (indicating that these cell lines have a deletion and a duplication). Therefore, the two different efficiencies reflect the configuration of the targeted *loxP* sites on the Chr 11 homologues in the parental cell lines, which is resolved into a deletion in *cis* and which is resolved into a deletion and a duplication in *trans* (8, 13). The *cis* events occurred several hundred times more efficiently than the *trans* events. To rule out any effect of HAT selection on Cre-mediated recombination, we transfected three *cis* double-targeted cell lines with the Cre expression plasmid and randomly picked colonies grown under no drug selection. We then determined the percentage of the colonies that were recombined by both drug resistance test and Southern analysis on individual clones. Of 279 colonies picked for all three double-targeted cell lines, 24 had undergone the 2-cM Cre-mediated deletion, yielding a Cre recombination efficiency of ~9%, which is not significantly different from that assessed by HAT selection. As a control, the 2-cM substrate with the mutant 3′ *hprt* cassette gave Cre recombination efficiencies of 0.007% for *cis* and 0.0001% for *trans* (8). Thus, the Cre recombination efficiency is improved by approximately 3 orders of magnitude after correction of the frameshifted 3′ *hprt* selection cassette.

Coupled Cre-*loxP* recombination and gene conversion. The *Hprt* cassette reconstructed by Cre-*loxP* recombination from the mutant 3′ *hprt* selection cassette should be nonfunctional. However, HAT-resistant colonies were readily obtained (8, 13). This raised the question as to the nature of the event that leads to the HAT-resistant colonies in these experiments. The frequency of spontaneous reversion is too low to explain the observed frequency of HAT-resistant clones from the mutant cassette. The frameshift mutation is located in a 2-kb overlap between the 5′ and the 3′ *hprt* cassettes, and therefore the mutation in the 3′ cassette may be corrected by homologous recombination with sequences in the 5′ cassette. We hypothesized that Cre brings the two *loxP* sites together to promote site-specific recombination and that during or immediately after this process the endogenous homologous recombination machinery repairs the mutation (Fig. 2B). This notion would predict that all recombination products would have the wild-type exon 3 sequence rather than a correcting single nucleotide insertion resulting from a spontaneous reversion. Sequence analysis demonstrated that all HAT-resistant colonies had acquired a wild-type sequence in the reconstituted full-length *Hprt* minigene (n = 10) (see Fig. 2B and Materials and Methods).

trans recombination events also generate an $Hprt^+$ deletion chromosome and the reciprocal product, a duplication chromosome, which retains the recombined overlapping region between the 5′ and 3′ cassettes (Fig. 2B). Sequence analysis of PCR products from exon 3 in the reciprocal product revealed that this exon 3 remained wild type in all cases analyzed (n = 17), indicating that the repair results from a gene conversion event (see Fig. 2B and Materials and Methods).

Long-range chromosomal rearrangements can be made in ES cells. Our chromosome engineering strategy has primarily focused on deletions, duplications, and inversions of a few centimorgans (8, 13). The ability to manipulate a larger region of the chromosome is desirable in many instances. For example, large inversions, when marked with a recessive lethal mutation, can be used as balancer chromosomes (2). ES cells with a large deletion may be useful in screens for recessive mutations in vitro. Since the apparent Cre recombination efficiency was dramatically increased with the corrected 3′ *hprt* cassette, we tested whether long-range (defined here as tens of megabases) deletions can be made in ES cells.

A deletion of 22 cM between *Hsd17b1* and *D11Mit69* on Chr 11 was used for this test. Previous attempts to generate this deletion in ES cells with the mutant 3′ *hprt* cassette had failed (8). The *D11Mit69* locus was targeted with the 3′ *hprt* cassette oriented for a deletion in an ES cell line that had been targeted at *Hsd17b1* (8). Fifteen double-*loxP*-targeted cell lines were transiently transfected with a Cre expression plasmid, and HAT-resistant colonies were counted after 12 days. Drug resistance tests indicated that four parental cell lines were double targeted in *cis* and eleven were double targeted in *trans*. The recombination events were confirmed to be Cre dependent because a mock transfection with a control plasmid (TyBS) yielded no HAT-resistant colonies for one *cis* and one *trans* double-targeted parental cell line. Unlike previous *cis-trans* tests, however, Cre recombination for both *cis* and *trans* configurations occurred at a similar efficiency of approximately

TABLE 1. Efficiency of Cre-mediated *loxP* site-specific recombination over different genetic distances

Interval (5′ *hprt*-3′ *hprt*)	Genetic distance (cM)	Cre recombination efficiency[a] (no. of colonies in HAT/no. of colonies with no drug)		
		loxP sites in opposite orientation: *cis* (inversion)[b] (*n*)	*loxP* sites in the same orientation	
			cis (deletion) (*n*)	*trans* (deletion-duplication) (*n*)
Hsd17b1-D11Mit199	2	ND	$(1.1 \pm 0.5) \times 10^{-1}$ (8)	$(4.7 \pm 0.7) \times 10^{-4}$ (5)
Hsd17b1-D11Mit69	22	ND	$(3.7 \pm 2.4) \times 10^{-5}$ (4)	$(3.1 \pm 1.1) \times 10^{-5}$ (11)
Wnt3-p53	24	$(2.2 \pm 0.6) \times 10^{-3}$ (5)	2.9×10^{-5} (1)[c]	$(8.2 \pm 0.9) \times 10^{-5}$ (5)
Hsd17b1-D11Mit142	30	$(3.2 \pm 0.7) \times 10^{-4}$ (3)	$(9.8 \pm 1.7) \times 10^{-6}$ (2)[c]	$(5.9 \pm 4.7) \times 10^{-5}$ (5)
Hsd17b1-D11Mit71	60	$(8.3 \pm 0.8) \times 10^{-5}$ (2)	$(9.5 \pm 2.1) \times 10^{-7}$ (2)[c]	$(1.4 \pm 0.2) \times 10^{-5}$ (4)

[a] Numbers are the means ± the standard deviations. ND, not determined.
[b] No colonies were obtained with the *trans* configuration because dicentric and acentric chromosomes are produced.
[c] Not confirmed by FISH analysis.

3×10^{-5} (Table 1 and see below). We further successfully generated a number of long-range rearrangements on Chr 11 (Fig. 3; Table 1). The most dramatic example is illustrated in Fig. 4D, where Cre recombination between two *loxP* sites targeted in *trans* to *Hsd17b1* and *D11Mit71* that are 60 cM away from each other on Chr 11 leads to a minideletion chromosome and a large duplication chromosome. Therefore, long-range chromosomal rearrangements, including deletions and deletion-duplications, can be generated with the improved selection cassette.

Large chromosomal deletions may cause ES cell lethality. The Cre-mediated deletion efficiency for the *cis* configuration differs by more than 3 orders of magnitude between a 2-cM (*Hsd17b1-D11Mit199*) and a 22-cM (*Hsd17b1-D11Mit69*) substrate (Table 1). The reduced Cre recombination efficiency for a larger substrate may simply reflect a lower efficiency of Cre-*loxP* juxtaposition with greater physical separation. However, it is also possible that ES cells with larger deletions may be selected against if the deletion has deleterious effects on cell viability or growth. In this scenario, only cells that have undergone a compensatory genetic change would survive. To test this, the deletion cell lines were analyzed by FISH with probes both internal and external to the deletion interval. Intriguingly, of five recombination products derived from three independent *cis* double-targeted parental cell lines, all were trisomy 11, with two wild-type and one deletion chromosome. The two wild-type chromosomes were found to exist as two separate chromosomes (as in Fig. 4B, three of five analyzed) or as a Robertsonian fusion in other cases (as in Fig. 4C, two of five analyzed, both of which derived from independent double-targeted parental cell lines). In contrast, the majority (three of four) of the *trans* recombination products analyzed contain the expected single deletion and duplication chromosomes. The remaining *trans* product contained a duplication chromosome and two deletion chromosomes in the Robertsonian configuration. All double-targeted parental cell lines analyzed, irrespective of the *cis* or *trans* configuration, contain two wild-type chromosomes (data not shown). These results indicate that the deletion in *cis*, which leads to a single copy of the 22-cM region of Chr 11, is haploinsufficient in ES cells. Consequently, rare variants are selected in which the remaining wild-type chromosome is duplicated. Thus, the hemizygous 22-cM deletion causes ES cell lethality or a severe growth disadvantage.

Cre-*loxP* recombination efficiency decreases over increasing genetic distances. The Cre recombination efficiency is an important consideration in designing Cre-*loxP*-based chromosome engineering experiments. To provide a framework for future experiments, we determined this efficiency for *cis* events at different genetic distances. Since a 22-cM deletion had been observed to cause cell death or a growth disadvantage, we assessed the efficiency of inversions as the indicator of Cre recombination efficiency for the larger intervals. Four rearrangements were included in this analysis: (i) a 2-cM deletion between *Hsd17b1* and *D11Mit199*, Del(11)4Brd; (ii) a 24-cM inversion between *p53* and *Wnt3*, In(11)8Brd; (iii) a 30-cM inversion between *Hsd17b1* and *D11Mit142*, In(11)6Brd; and (iv) a 60-cM inversion between *Hsd17b1* and *D11Mit71*, In(11)7Brd (Fig. 3). When the two *loxP* sites are in opposite orientations, approximately half of the independent double-targeted cell lines give HAT-resistant colonies (interpreted as *loxP* sites in *cis*), and the other half do not give any colonies (interpreted *loxP* sites in *trans*), presumably due to the formation of dicentric and acentric chromosomes. FISH analysis confirmed that the relevant inversions had occurred in representative clones from all three large genetic intervals (data not shown). As shown in Fig. 5, between 2 and 60 cM, the logarithm of the Cre recombination efficiency is inversely proportional to the genetic distance between the *loxP* sites.

Tissue-specific chromosome engineering. Several deletions of a few centimorgans around the *Hsd17b1* locus on Chr 11 are heterozygous lethal (8). Although this underscores the developmental importance of this chromosomal region, lethal deletions cannot be used for genetic screens. However, if the de-

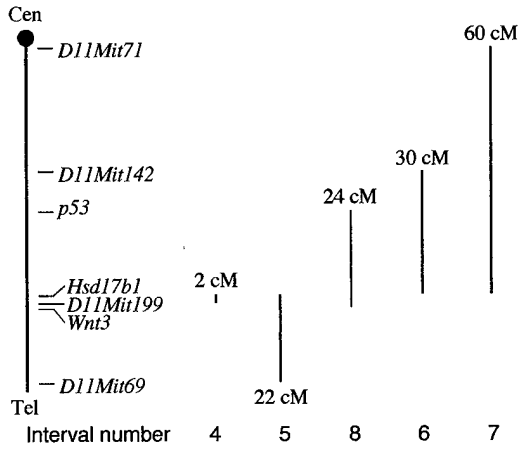

FIG. 3. Genetic intervals of rearrangements made on mouse Chr 11 in this study. 2 cM, *Hsd17b1-D11Mit199*, deletion, deletion-duplication; 22 cM, *Hsd17b1-D11Mit69*, deletion, deletion-duplication; 24 cM, *Wnt3-p53*, inversion, deletion-duplication; 30 cM, *Hsd17b1-D11Mit142*, inversion, deletion-duplication; 60 cM, *Hsd17b1-D11Mit71*, inversion, deletion-duplication. The total genetic distance from centromere (Cen) to telomere (Tel) is about 80 cM.

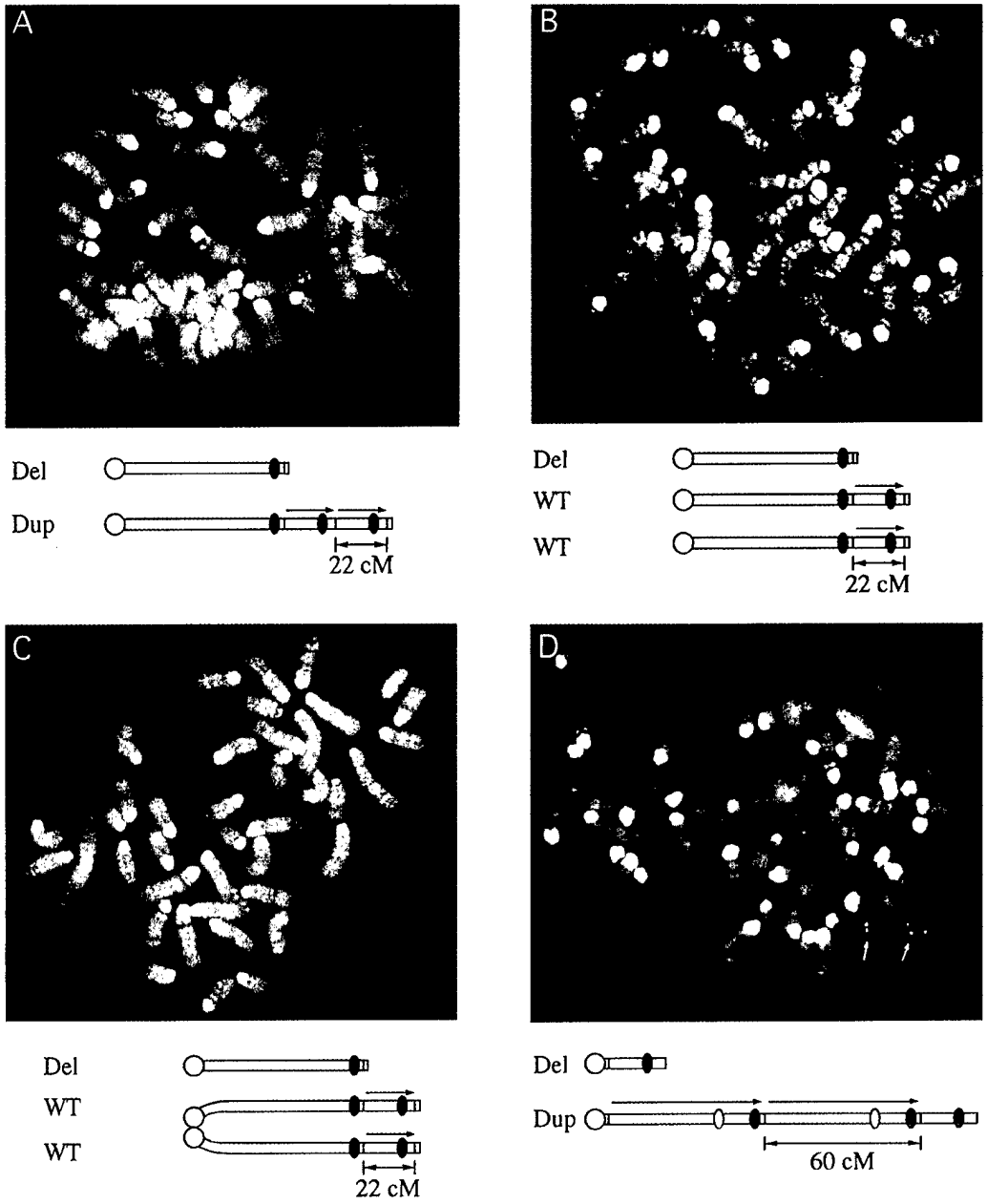

FIG. 4. FISH analysis of long-range Cre recombination products on Chr 11. (A) Del(11)5Brd-Dp(11)5Brd, a 22-cM deletion chromosome and a 22-cM duplication chromosome produced by a *trans* event between *Hsd17b1* and *D11Mit69*. (B) Del(11)5Brd-WT-WT, a 22-cM deletion chromosome produced by a *cis* event between *Hsd17b1* and *D11Mit69*, while the remaining wild-type chromosome is duplicated to survive. (C) The same as in panel B except that the duplicated wild-type chromosomes are in a Robertsonian (or iso-chromosome) configuration. (D) Del(11)7Brd-Dp(11)7Brd, a 60-cM deletion chromosome and a 60-cM duplication chromosome produced by a *trans* event between *Hsd17b1* and *D11Mit71*. Colors: yellow, BAC 232M23 (*D11Mit320*); red, BAC 330H2 (*D11Mit263*); green, BAC 330P14 (*D11Mit11*). Two or more probes were differentially labeled and artificially colored.

letion can be made somatically, for instance, in a tissue- or cell-type-specific manner, the problem of heterozygous lethality can be partially circumvented. To test this possibility, we generated a 2-cM *Hsd17b1-D11Mit199* double-targeted mouse line (deletion substrate) and crossed it to a cardiac-specific Cre (i.e., αMyHC-Cre) (1). The αMyHC-Cre line had previously been used to make cardiac-specific deletions of several kilobases with an efficiency of up to 90% (1). Tissue DNA was isolated from two progeny that inherited both the αMyHC-Cre transgene and the 2-cM substrate. PCR analysis with primers specific to the reconstituted *Hprt* minigene was performed to determine whether the Cre-mediated recombination had occurred (Fig. 6A). This analysis demonstrated that the Cre recombination occurred in heart, but not in skeletal muscle, liver, lung, or spleen (Fig. 6A). To provide a more quantitative measure of Cre recombination, Southern analysis was performed on two animals by using restriction digestions and a probe at *Hsd17b1* that would distinguish the wild-type allele, the (double) targeted allele and the Cre-recombined allele (Fig. 6B). Deletion occurred exclusively in the heart but not in the other organs tested (Fig. 6B). Based on the ratio of intensity of the recombined fragment and the predeletion allele for

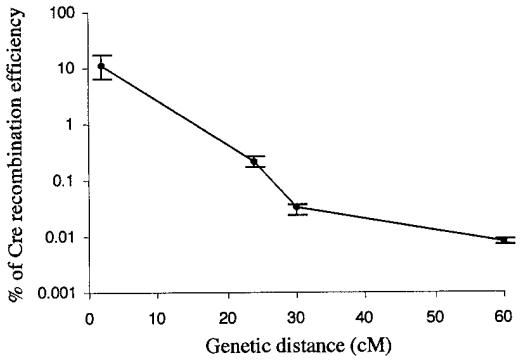

FIG. 5. Efficiency of Cre recombination over genetic distance. The percentage of Cre recombination efficiency (*y* axis, in $\log_{10}$ scale) is plotted against the genetic distance (*x* axis, in linear scale). The first datum point represents a deletion. The other three points with larger genetic distances represent inversions. Error bars indicate the standard deviations. The numbers of independent experiments were as indicated in Table 1.

both animals tested, the deletion efficiency in the heart is about 10%.

DISCUSSION

The organism that the Cre-*loxP* system is derived from, bacteriophage P1, evolved the system to resolve its ~100-kb genome into monomeric circular forms (18). The Cre-*loxP* site-specific recombination system has been extensively used for conditional genetic technology, namely, the temporal and spatial control of gene expression in mice (16). In these applications, the genetic material involved (as determined by the distance between the two *loxP* sites) is usually a few kilobases. We have previously shown that this system can be adapted for substrates of several megabases by incorporating a positive selection scheme (8, 13). In the present study, we redefined the Cre recombination efficiency for a 4-Mb substrate, after we corrected a mutation in the selection cassette. Surprisingly, the efficiency for this substrate is approximately 11% by transient Cre expression. This efficiency approaches that obtained with substrates of several kilobases and indicates that at between several kilobases and several megabases the Cre-*loxP* recombination occurs at comparable efficiencies. This might reflect aspects of chromatin domain organization such that sequences that are 1-kb to 1-Mb apart may have similar separations in three-dimensional space. In this aspect, the fact that the 2-cM cardiac-specific deletion can be detected by Southern analysis is of particular significance. In many cancers, interstitial deletions are the dominant mode for loss of the remaining allele of a tumor suppressor gene (6). Therefore, in vivo chromosomal deletions can be used to mimic somatic LOH in human cancers and in searches for novel tumor suppressor genes in combination with point mutagenesis.

The 22-cM deletion between *Hsd17b1* and *D11Mit69* on Chr 11 appears to cause ES cell lethality or a severe growth disadvantage because deletion products for this interval exclusively carry an additional wild-type Chr 11. This may be due to a dosage effect of one or multiple genes in this interval such that a single copy of these genes cannot support the normal growth of ES cells (haploinsufficiency). The Cre-*loxP*-mediated deletion of this 22-cM region therefore selects for cells that have duplicated the wild-type Chr 11. This result underscores the tight control of the euploid ES cell genome. A region of haploinsufficiency has also been proposed to reside on Chr 9 in studies on a radiation-induced deletion complex (19). The observation of haploinsufficiency in ES cells is in direct contrast with many cancer cells that often carry large chromosomal deletions and chromosomal losses. Such a unique feature of ES cells may be further studied by isolating suppressors of this lethality caused by the deletions. On the other hand, these data indicate that duplications are tolerated better than deletions in ES cells. This is consistent with the notion that monosomies rarely, if ever, exist, whereas trisomy 8, 11, and 15 and several other chromosomes have been observed in ES cells (9). The relatively frequent occurrence of trisomy 11 is further suggested by our observation that one of four 22-cM deletion-duplication products analyzed by FISH contain one duplication and two deletion chromosomes where the deletion chromosome is presumably not required to be duplicated for cell survival or growth. The lethality caused by large deletions in ES cells precludes a straightforward approach of using the deletion as a partial haploid reagent in mutagenesis screens.

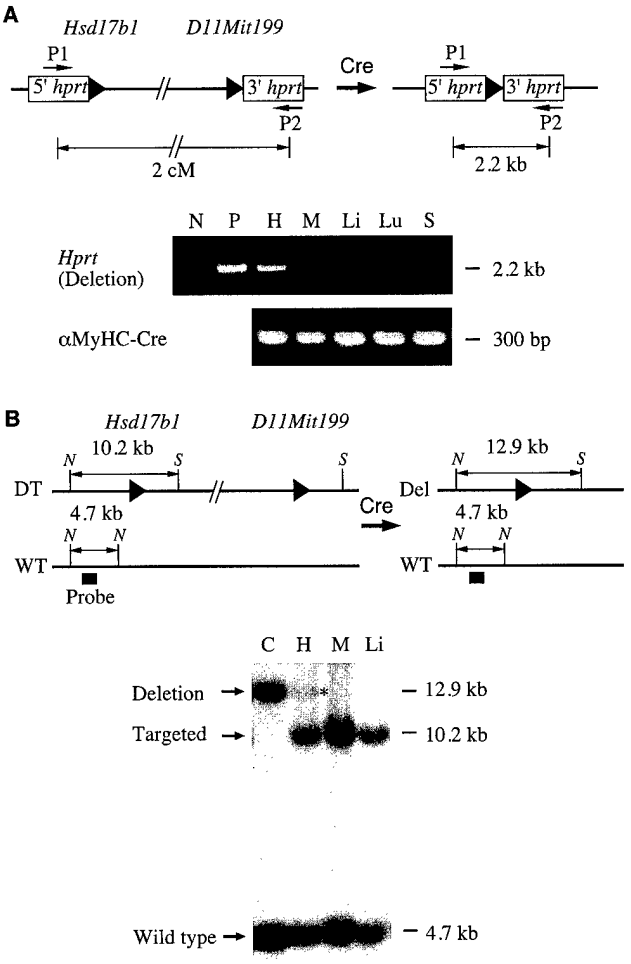

FIG. 6. Cardiac-specific 2-cM deletion between *Hsd17b1* and *D11Mit199*. (A) PCR analysis of the deletion products. P1 and P2, primers used to specifically amplify the full-length *Hprt*. PCR reactions on αMyHC-Cre serve as a control. N, negative control; P, positive control; H, heart; M, skeletal muscle; Li, liver; Lu, lung; S, spleen. (B) Southern analysis of the deletion products. C, control cell line that contains a deletion and a wild-type chromosome. The cardiac-specific deletion band is indicated by an asterisk. *N*, *NheI*; *S*, *SfiI*; solid triangle, *loxP* site.

It is possible that the partial-trisomy ES cells selected by the 22-cM *cis* deletion are derived from an underlying trisomy 11 population in the ES cells transfected with Cre. Although these cells are not detected by analysis of double-targeted clones,

extrapolation of the inversion recombination efficiencies suggests that either these cells are present at 10^{-2} frequencies in the transfected clones or that this nondisjunction event is induced by the Cre-*loxP* recombination event itself.

Large-deletion-associated ES cell lethality can obscure the Cre recombination efficiency. We therefore determined the Cre efficiency by using large inversion substrates. This analysis indicates that Cre recombination efficiency decreases over increasing genetic distances. However, in all cases, the recombination products (HAT-resistant colonies) are readily obtained in sufficient numbers in a single experiment except when inviable products are generated (dicentric and acentric chromosomes). For multimegabase substrates, the logarithm of the Cre recombination efficiency is approximately inversely proportional to the genetic distance (Fig. 5). This can be used as a guide for future experiments with Cre-*loxP*-based chromosome engineering. However, other factors, such as chromosomal locations and differences in experimental manipulations, may affect the Cre recombination efficiency. For deletions, the Cre recombination drops more precipitously as the genetic distance increases for two reasons. First, the physical barrier Cre has to overcome to bring the two *loxP* sites together is greater as the distance between the two *loxP* sites increases, as in inversions. Second, larger deletions may cause ES cell lethality or a growth disadvantage and are consequently selected against after Cre recombination. In the *trans* configuration where a deletion and a duplication chromosomes are the products, Cre recombination efficiency is moderately reduced with an increasing genetic distance (Table 1). This suggests that chromosome homologues may pair in a mitotic cell cycle, assisting Cre recombination by bringing the two *loxP* substrates on different chromosomes to the same subcellular location. Under such circumstances, the closer the two *loxP* sites are genetically, the closer they are physically when the two chromosomes pair, and therefore, the higher the Cre recombination efficiency. Alternatively, if *trans* recombination occurs mainly in G_2 and recombined sister chromatids tend to segregate away from each other, as reported in *Drosophila* (4), the HAT-resistant deletion products will frequently contain a wild-type chromosome instead of the duplication chromosome. In this scenario, *trans* deletion-duplication events involving a larger distance will appear to occur at a lower frequency due to the production of haploinsufficient deletions.

The Cre recombination efficiency for large deletion-duplications is probably comparable to that for translocations between nonhomologues. In some of our experiments, we analyzed some random integration clones when targeting the second *loxP* site. Upon Cre expression, approximately half of these clones give HAT-resistant colonies and the other half do not give viable HAT-resistant colonies. The former group presumably yields translocations, while the latter group yields dicentric and acentric products. The efficiency of generating these translocations is about 10^{-5}. It has been reported using a similar strategy that Cre recombination efficiency for a translocation between chromosomes 12 and 15 occurs at about 10^{-7} (17). The higher efficiency in our experiments may be due to the Cre plasmid, the tissue culture conditions, and/or the electroporation procedures used. It remains possible that the 2-kb homology between our 5′ *hprt* and 3′ *hprt* cassettes assists the Cre-*loxP* recombination by recruiting the homologous recombination machinery to help secure the *loxP* site recombination synapse.

The mutant 3′ *hprt* cassette used in previous chromosome engineering experiments provides a unique opportunity for studying a potential interaction between homologous and site-specific recombination. Sequence analysis of Cre recombination products indicates that the mutation is repaired by homologous recombination with the wild-type template in the 5′ *hprt* cassette. This homologous recombination cannot occur in the absence of site-specific recombination since the homology is only 2 kb, but the substrates are on different chromosomes or far away (multimegabases) from each other on the same chromosome. Therefore, it must have occurred during or immediately after the Cre-*loxP* recombination. It is possible that the Holliday junction structure created by Cre (21) can be resolved by homologous recombination machinery. This scenario would suggest that the two recombination events are not mutually exclusive and can be coupled under specific circumstances. The other possibility is that Cre-*loxP* recombination facilitates gene conversion merely by bringing the two substrates together. Immediately after site-specific recombination, homologous recombination occurs. Since HAT-resistant colonies for a 2-cM substrate are obtained with an efficiency of approximately 3 orders of magnitude higher with the wild-type 3′ selection cassette than with the mutant version, homologous recombination responsible for repairing the mutation occurs ca. 0.1% of the time after Cre recombination.

Taken together, the Cre-*loxP* chromosome engineering strategy provides a powerful tool for genetic studies and for genome manipulation. We explored the possibility and determined the efficiency of generating various chromosomal rearrangements on mouse Chr 11. We conclude that any desired rearrangement can be made with the Cre-*loxP* system provided that the rearrangement does not have any deleterious effect on the ES cells. Cre-*loxP* recombination is very efficient for substrates of a few centimorgans both in tissue culture and in vivo. This efficiency decreases over increasing genetic distances between the two *loxP* sites. The work presented here provides a framework for future applications of chromosome engineering.

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